The AAPS Journal 2007; 9 (1) Article 5 (http://www.aapsj.org).

Themed Issue: Bioanalytical Method Validation and Implementation: Best Practices for Chromatographic and Ligand Binding Assays
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The History of Bioanalytical Method Validation and Regulation: Evolution of a Guidance Document on Bioanalytical Methods Validation

Submitted: January 17, 2007; Accepted: January 29, 2007; Published: February 16, 2007

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Introduction

Bioanalytical method validation (BMV) employed for the quantitative determination of drugs and their metabolites in biological fluids plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence, pharmacokinetic, and toxicokinetic study data. These studies generally support regulatory filings. The quality of these studies is directly related to the quality of the underlying bioanalytical data. It is therefore important that guiding principles for the validation of these analytical methods be established and disseminated to the pharmaceutical community. This chapter provides historical perspectives in the evolution and development of the BMV guidance. This guidance, virtually in one form or another, has been adopted universally as a standard procedure for validating bioanalytical assays used for pharmacokinetic, bioavailability, and bioequivalence studies intended for regulatory submission.

First Bioanalytical Method Workshop

Analytical methods employed for the quantitative determination of drugs and their metabolites in biological samples must generate reproducible and reliable data in order to permit valid interpretation of the studies they support. It is essential to employ well-characterized and fully validated analytical methods to yield reliable results that can be satisfactorily interpreted. It is recognized that analytical methods and techniques are constantly undergoing changes and improvements; and in many instances, they are at the cutting edge of the technology. It is also important to emphasize that each analytical technique has its own characteristics, which will vary from analyte to analyte—in these instances, specific validation criteria may need to be developed for each analyte. Moreover, the appropriateness of the technique may also be influenced by the ultimate objective of the study.

While validation of each method will stand on its own, there may be situations when comparison of methods will be necessary (eg, when more than one method has been employed in a long-term study). When sample analysis for a given study is conducted at more than one site, it is necessary to validate the analytical method(s) at each site and provide appropriate validation information for different sites to establish interlaboratory reliability. Unless a method is used on a regular basis to provide confidence in its continued validity, it is essential to document that the method is still valid prior to analysis of samples in a study.

Before the first BMV workshop, there was a lack of uniformity in conducting validation of bioanalytical methods and submission of data to regulatory agencies. The bioanalytical validation workshop in 1990 was the first major workshop dedicated to investigating and harmonizing procedures required in method validations. The workshop was cosponsored by the American Association of Pharmaceutical Scientists (AAPS), the United States Food and Drug Administration (FDA), the International Pharmaceutical Federation (FIP), the Health Protection Branch (HPB), and the Association of Analytical Chemists (AOAC). The conference focused on requirements for bioanalytical methods validation, procedures to establish reliability of the analytical method, parameters to ensure acceptability of analytical method performance, method development (prestudy validation), and method application (in-study validation). The workshop defined essential parameters for BMV—accuracy, precision, selectivity, sensitivity, reproducibility, limit of quantification, and stability—and addressed "how to" evaluate and determine these parameters. In addition to defining various bioanalytical method validation parameters, the workshop discussed appropriate method validation procedures and defined the standard curve, recovery, and replicate analysis. It was clarified that it is not essential to have 100% recovery, but it is important that the recovery be reproducible. It was also stressed that in most of the chromatographic assays, duplicate or replicate analysis was not needed as it did not add any value to the analytical results. The workshop clearly identified 2 distinct phases of BMV: (1) analytical method development (prestudy validation), where the appropriate bioanalytical method with its various parameters is developed, and the assay is defined; and (2) application of the bioanalytical method to actual analysis of samples from bioavailability, bioequivalence, and pharmacokinetic studies. One of the most important outcomes of the first workshop was that it defined "the acceptance criteria for a run."

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The workshop was attended by more than 575 scientists from around the world. The workshop report was published in *Pharmaceutical Research*[1] and in 4 additional journals for better dissemination of the information. The workshop report served well as an industry outline for practice by bioanalytical laboratories and as a reference for regulatory agencies worldwide. The report provided guiding principles for BMV, defined validation parameters, provided specific recommendations for BMV, provided acceptance criteria for analytical runs, and suggested a priori establishment of a protocol for repeat analysis. The workshop brought all relevant issues related to bioanalytical methodology together and provided a platform for scientific discussions and deliberations. It raised awareness among scientists that validated analytical methods were needed if in vivo data were to be accepted.

Although the workshop addressed bioanalysis in general, it acknowledged the differences between chromatographic and ligand binding (nonchromatographic based) methods. The workshop also brought about the convergence of views of stake holders—industry, contract research organizations, academia, and regulatory scientists—and established general standards for acceptance criteria for BMV. The outcome of the first workshop and its report resulted in improved quality of data submissions to regulatory authorities.

Draft Bioanalytical Methods Validation Guidance

The first workshop was well received within the global pharmacokinetics community. The need to validate bioanalytical methods and the development and acceptance of general standards for their conduct, brought about significant improvements in the quality of bioanalytical methods and in the submission of pharmacokinetic, bioavailability, and bioequivalence studies to the FDA. Subsequently, several national and international conferences were held to discuss the first workshop report. However, the workshop report was not an official document of the FDA. Therefore, the agency decided to develop and publish a draft guidance in January 1999.[2] The draft guidance was primarily based on the first workshop report and the experience gained by the agency since the first workshop. The intentions here were to seek public comment before finalizing the BMV Guidance.

Second Bioanalytical Method Workshop

The second workshop was cosponsored by AAPS and the FDA and was held in January 2000, 1 year after the publication of the draft guidance by the agency. This workshop provided stimulus and opportunity for scientists to air their views and share their experiences over the 10 years since the first workshop report on analytical methods validation was issued. This forum also offered the opportunity for scientists to comment on the draft guidance, which had further helped to produce consistency, efficiency, and scientific validity in the procedures used in validating and implementing analytical methods within the industry.

The workshop focused on discussing the advances in analytical technology that had occurred over the past decade and reconfirmed and updated the principles of BMV. There had been significant advancements in the field of mass spectrometry, with the development of new interfaces and ionization techniques. These advancements resulted in the rapid emergence and widespread commercial use of "hyphenated" mass spectrometry-based assays (eg, liquid chromatography-mass spectrometry-mass spectrometry [LC-MS-MS]), which have largely replaced conventional high-performance liquid chromatography (HPLC), gas chromatography (GC), and GC-MS assays. During this time, the use of multi-well plates, automated robotic sample processing, and electronic data reporting became very common. Given the certainty of continued technological advances, the future will very likely bring new, even more powerful bioanalytical approaches as the search for more rapid throughput and increased sensitivity continues.

Despite these widespread changes in technology, there remains a need for clearly defined validation criteria for bioanalytical methods intended for analysis of each analyte (drug and/or metabolites) in various biological matrices. In the case of "hyphenated" mass spectrometry techniques, such as LC-MS-MS, there are unique requirements that demand attention. The second workshop discussed the advances in "hyphenated" mass spectrometry and ligand binding assays. Selectivity issues in ligand binding assays were discussed in detail. Ligand binding assays must be selective for the analyte. Two types of issues must be considered: (1) interference from substances that are physicochemically similar to analyte (eg, metabolites, endogenous compounds) and (2) interference from matrix components (also termed "matrix effects") that are unrelated to the analyte. In ligand binding assays, standard curves are inherently nonlinear and require more concentration points to define the curve. Accuracy in these types of assays may be improved by the use of replicate (duplicate or even triplicate) sample analysis. However, the same procedures must be followed for unknown samples.

The second workshop also discussed different categories of validation; namely, Partial Validation, Cross-Validation, and Full Validation. The workshop reemphasized that it is not necessary to have 100% recovery, but it is important to have reproducible and consistent recovery when using an extraction procedure. The importance of standard curve and quality control acceptance criteria were reemphasized. This workshop resulted in the report "A revisit with a decade of progress"[3] and formed the basis for FDA Guidance on Bioanalytical Methods Validation (May 2001).[4]

FDA Guidance on Bioanalytical Method Validation

Bioanalytical method validation includes all of the procedures required to demonstrate that a particular bioanalytical method for the quantitative determination of the concentration of an analyte (or series of analytes) in a particular biological matrix is reliable for the intended application. The most widely employed bioanalytical techniques include, but are not limited to, conventional chromatographic-based methods (such as GC and HPLC), mass spectrometry-based methods (such as GC-MS and LC-MS), and ligand-based assays (such as radioimmunoassay [RIA] and enzyme-linked immunosorbent assay [ELISA]). Many of the principles, procedures, and requirements for quantitative bioanalytical method validation are common to all types of analytical methodologies. Some of the key elements of the final FDA Guidance on BMV[4] are given below. The reader is advised to review the guidance for more details.

Bioanalytical Method Development and Establishment (chemical assays)

The following principles of bioanalytical method validation provide steps for the development of a new method or for establishing an existing method. The parameters essential to ensure the acceptability of the performance of a bioanalytical method are accuracy, precision, selectivity, sensitivity, reproducibility, and stability. The guidance provides "how to" information for determining these parameters. The stability of the analyte in biological matrix at intended storage and operating conditions should be established. The guidance also establishes the requirements for a standard curve. The matrix-based standard curve should consist of a minimum of 5 standard points, excluding blanks, using single or replicate samples, and should cover the entire range of expected concentrations. All these parameters need to be defined during the Full Validation of a bioanalytical method. The lower limit of quantitation (LLOQ) should serve as the lowest concentration on the standard curve and should not be confused with the limit of detection (LOD). There are 2 distinct phases of BMV: (1) the bioanalytical method development phase in which the assay is defined and validated, and (2) the application to actual analysis of samples from pharmacokinetic, bioavailability, bioequivalence, and drug interaction studies. Quantification of metabolites and of stereoisomers, if and when considered necessary, should follow an identical protocol for validation including accuracy, precision, selectivity, sensitivity, reproducibility, and stability.

Application of a Validated Bioanalytical Method to Routine Drug Analysis

The routine analysis of biological samples can be done by single determination without a need for duplicate or replicate analysis. A matrix-based standard curve should be generated for each analytical batch for each analyte and should be used for calculating the concentration of analyte in the unknown samples assayed with that run. It is important to use a matrix-based standard curve that will cover the entire range of concentrations in the unknown samples. Estimating the concentration of unknowns by extrapolating the standard curve below the LLOQ or above the upper limit of quantitation (ULOQ) is not recommended. Instead, it is recommended that the standard curve be redetermined or samples be re-assayed after dilution with the matrix. The matrix-based quality control (QC) samples spiked with analyte should be used to accept or reject the run.

Bioanalytical Methods Validation for Microbiological and Ligand-based Assays

Selectivity Issues

As with chromatographic methods, ligand binding assays must be shown to be selective for the analyte. Two types of selectivity may be considered: (1) "Specific" (interference from substances that are physicochemically similar to the analyte), and (2) "Nonspecific" (also termed "matrix effects"; interference from matrix components that are unrelated to the analyte, such as from homolysis, serum proteins, lipemia, etc).

Quantification Issues

Immunoassay standard curves are inherently nonlinear and, in general, require more concentration points to define the fit over the standard curve range than do chemical assays. For all assays, the key factor is the accuracy of the *reported results*. This accuracy may be improved by the use of replicate (duplicate or even triplicate) sample analysis. In the case where replicate analysis has to be performed to improve accuracy, the same procedure must be followed for unknown samples.

Validation

It is accepted that during the course of a typical drug development program, a defined bioanalytical method will undergo many modifications. These evolutionary changes (eg, addition of a metabolite, lowering of the LLOQ) require different levels of validation to demonstrate continuity of the validity of an assay's performance. Three different levels/types of method validations, Full Validation, Partial Validation, and Cross-validation, are defined and characterized as follows.

Full Validation

Full Validation is necessary when developing and implementing a bioanalytical method for the first time for a new drug entity. If metabolites are added to an existing assay for

quantification, then Full Validation of the revised assay is necessary for all analytes measured.

Partial Validation

Partial Validations are modifications of validated bioanalytical methods that do not necessarily require full revalidations. Partial Validation can range from as little as 1 assay accuracy and precision determination to a "nearly" Full Validation. Typical bioanalytical method changes that fall into this category include, but are not limited to, bioanalytical method transfers between laboratories or analysts, instrument and/or software platform changes, change in species within matrix (eg, rat plasma to mouse plasma), changes in matrix within a species (eg, human plasma to human urine), change in analytical methodology (eg, change in detection systems), and change in sample processing procedures.

Cross-validation

Cross-validation is a comparison of 2 bioanalytical methods. Cross-validations are necessary when 2 or more bioanalytical methods are used to generate data within the same study. For example, an original validated bioanalytical method serves as the "reference" and the revised bioanalytical method is the "comparator." The comparisons should be done both ways. Cross-validation with spiked matrix and subject samples should be conducted at each site or laboratory to establish interlaboratory reliability when sample analyses within a single study are conducted at more than 1 site, or more than 1 laboratory, and should be considered when data generated using different analytical techniques (eg, LC-MS-MS vs ELISA) in different studies are included in a regulatory submission.

Acceptance Criteria for the Batch

- Standards and QC samples can be prepared from the same spiking stock solution, provided the solution stability and accuracy have been verified. A single source of matrix may also be used, provided selectivity has been verified.
- Standard curve samples can be positioned anywhere in the run.
- Matrix-based standard calibration samples: 75%, or a minimum of 6 standards, when back-calculated (including ULOQ) should fall within ±15% of nominal, except for LLOQ when it should be within ±20% of the nominal value.
- Quality-control samples: replicate quality-control samples, (at least duplicated), at a minimum of 3 concentrations (1 within 3× of the LLOQ, low QC; 1 in the midrange, middle QC; and 1 approaching the high end of the range, high QC) should be incorporated into each run. The results of the QC samples provide the basis of accepting or rejecting the run. At least 67% (4/6) of the QC samples must be within 15% of their respective nominal (theoretical) values; 33% of the QC samples (not all replicates at the same concentration) may be outside the ±15% of the nominal value.

Conclusions

The FDA Guidance[4] represents the final synthesis of the 2 workshops held in 1990 and 2000, with the Draft Guidance providing a constructive and building bridge between the 2 important workshops. The final Guidance published in 2001 incorporates most of the recommendations from the workshop reports.

The Guidance represents the Agency's best scientific judgment and current thinking at the time of guidance development. It is a living document and may need to be updated as more information is available. The Guidance is an informal and nonbinding document and does not create or confer any rights for or on any person and does not operate to bind the FDA or the public. An alternate approach may be used if such approach satisfies the requirements of the applicable statute, regulations, or both.

The Guidance provides consistency in procedure. It reduces differences in approach within the industry concerning validation and implementation of analytical methods. It also makes the process efficient and technically valid. It is important to note that no conference report or guidance can cover all issues and/or *allwhat if's,* and there is no substitute for common sense. Each issue should be evaluated in full light of the objectives and aims of analysis, scientific basis, and proof for deviation or anomalous observation. There is no substitute for "Good Science." The Guidance can provide only the guiding principles.

Since bioanalytical methods underpin the appropriate evaluation and interpretation of pharmacokinetic, bioavailability, and bioequivalence study data, the integrity of the bioanalytical method should be maintained. The FDA Guidance helps in guiding the scientists in the right direction to develop an appropriately validated bioanalytical method for submitting to regulatory authorities.

Bioanalytical tools and techniques continue to evolve. In addition, significant scientific and regulatory experience has been gained since the last workshop in 2000 and issuance of the Guidance in 2001. The evolution and expansion of bioanalytical tools require a critical review of the scope, applicability, and success of the presently employed bioanalytical guiding principles. To address this, the third workshop in the series was held in May 2006. The purpose of this workshop was to identify, review, and evaluate problems,

common practices, the existing Guidance, white papers, and articles on the subject. The focus of the workshop was primarily on quantitative bioanalytical methods' validation and their use in sample analysis, focusing on chromatographic and ligand binding assays. A report of this workshop is in this theme issue of *The AAPS Journal*.[5]

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